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3. AMERICAN JOURNAL OF OTOLOGY, (2000 Mar) 21 (2) 161-7.
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CHAPTER 12

Mitochondrial DNA Mutations and Heart Disease

Takayuki Ozawa and Mika Hayakawa

Introduction

Mitochondrial (mt) DNA encodes important subunits of the mt oxidative phosphorylation system—the central cellular apparatus for bioenergy production. Mutations in mtDNA have been shown to be a crucial causative factor of myocardial dysfunction, hence this is called mt cardiomyopathy (CM).[1] The majority of CMs present clinical manifestations of either dilated or hypertrophic CM (DCM or HCM, respectively). Kelly and Strauss[2] classified CM into two general categories: (1) disorders of cardiac energy metabolism, which include defects in fatty-acid oxidation and disorders of mt oxidative phosphorylation; and (2) abnormalities of myocardial contractile and structural proteins, which include familial HCM and X-chromosome linked muscular dystrophies. However, based on the molecular genetics and pattern of inheritance, it is more logical to classify CM into the following two groups: (i) familial CM linked with nuclear (n) DNA mutations, which are inherited as autosomal dominant/recessive disorders in 50-56% of HCM,[3] and in 20-25% of DCM;[4] and (ii) mtCM caused by mtDNA point mutations, which are inherited from an affected mother but not from an affected father (maternal inheritance) or are of sporadic occurrence, and by somatically acquired mtDNA deletions. In a similar way, diabetes mellitus (DM) caused by mtDNA mutations is called mitochondrial DM (mtDM).[5] The nucleotide position (np) 3243 A→G point mutation in the $tRNA^{Leu(UUR)}$ gene has been reported to be associated with DM in about 1.5% of the diabetic population.[5] Often, patients with mtCM harboring the np3243 mutation as well, express signs of insulin-deficient type DM (Fig. 12.1, Table 12.1). Clinically, these patients have classified as "diabetic CM".[6]

Following the complete sequencing of human mtDNA,[7] there were reports on the abnormality of mtDNA gene products in patients with encephalomyopathies.[8-9] These were followed by reports on mtDNA mutations associated with degenerative diseases and aging.[10-13] In 1989 we proposed[14] that the accumulation of somatic mtDNA mutations during a lifetime may be major factor in human aging and degenerative diseases. This proposal was based on the following observations: (i) a high frequency of gene mutation found in mtDNA; (ii) the small size of the mt genome and its known information content; (iii) the inefficient repair mechanism for mtDNA; and (iv) the somatic segregation of individual mtDNA during

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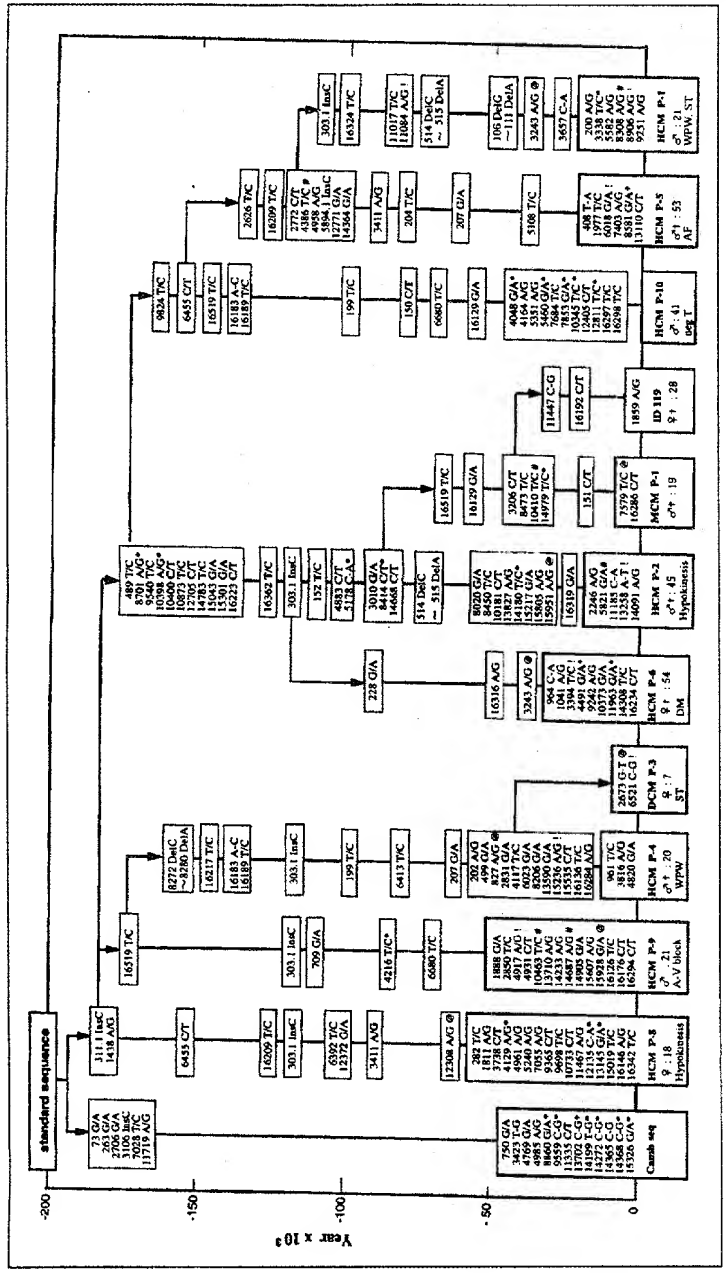

eukaryotic cell division. Since then, the practical use of polymerase chain reaction (PCR) technology[15] has led to numerous reports documenting an extensive array of age-dependent accumulation of large deletions spanning several genes.[13,16-20]

Together with the observation of mutations, it was also found that the cumulative increase in oxidative damage in muscle mtDNA correlates with the increase in deleted (Δ) mtDNA associated with aging and mtCM[1,20,21] (Fig. 12.2). This indicated that the oxidative damage in mtDNA may accelerate somatic mutations, as suggested by Harman in his "free radical theory of aging."[22] Therefore, unifying both ideas of the mitochondrial and free radical theory of aging, the "redox mechanism of aging"[23] was proposed as the molecular basis for the progressive decline of cellular bioenergy and naturally occurring cell death (apoptosis), resulting in degeneration and atrophy associated with age and with degenerative diseases (e.g., muscle weakness of senescence, declining mental capacity, age-related progressive decline of ventricular performance[24]).

Both point and deletion mutations in mtDNA were reported to be the cause of degenerative diseases.[25] However, the cause-effect relation between the mutations and the clinical phenotype remained unclear in these early studies. This was due to the fact that the mutation survey was carried out within a limited region of mtDNA. Since then, accumulated data with the base-sequencing of the entire mtDNA[26] clarified a dominant feature of the mtDNA diseases and suggested that the clinical signs and symptoms are triggered by maternally inherited or somatically acquired[27] point mutations located in many genes[28] (Fig. 12.1). It is clear that the mutational genotypes, which depend on the severity of point mutations and their combination, correspond to the clinical phenotypes, ranging from the asymptomatic to incapacitating symptoms (Fig. 12.1). The combination of point mutations extensively accelerates the accumulation of somatic mutations of mtDNA.[23] An entire picture of the mutational genotype is now possible through the recently devised total detection system (TD system).[29] The TD system has documented the extensive fragmentation of mtDNA into hundreds of ΔmtDNA in pediatric mtCM patients as well as senescent individuals[30-32] (Fig. 12.3, Table 12.2). The fragmentation could be mimicked within 3 days in cultured fibroblasts under oxygen stress.[33] The somatic mutations lead to various cellular defects—in components of the mt electron transfer chain (ETC),[34-35] decreased oxygen utilization by

Fig. 12.1 (opposite). Clustering of point mutations in mtDNA in patients with mtCM. From the total base-sequencing of 65 individuals including 32 mtCM patients, the base-sequence of mtEve is deduced. Divergence of the Camb Seq from the mtEve is also deduced from our data-base. Among the mtCM patients, subsequently diverged base-substitutions are demonstrated together with their nucleotide positions. The base-substitutions including several point mutations are illustrated by abbreviations: /, transition; -, transversion; Ins, insertion of nucleotide; Del, deletion of nucleotide; *, mutation that changes amino acid; !, mutation that changes evolutionarily conserved amino acid among six species of mammalian; #, substitution of nonconserved base in tRNA gene; @, mutation in the tRNA gene or in the rRNA gene. The base-substitutions unique to the patient are enclosed with the identification of the patient (P) with sex, age of death (†) or of genetic examination, and major complications. HCM: Hypertrophic cardiomyopathy. DCM: Dilated cardiomyopathy. MCM: Mt cardiomyopathy. ID: Identification number for the regal anatomy. HCM, P-9 is Australian of Greek-origin, HCM, P-8 is Australian of English-descent, and others are Japanese. Modified from ref. 23.

Table 12.1. Genotypes and phenotypes of the patients with mtCM

Genotypes	Patients	Sex/Age	Mit⁻	Mit⁻⁻	Syn⁻	Sympts	Finds	Arrhyths	Complts
I Mit⁻	HCM P-3	m, alive 25	AP6, CO_3			CTR 47	neg T		ret pig
	HCM P-10	m, alive 41	AP6, N3, N1, N2, CO_2, N3, N5			CTR 54	neg T		
	HCM P-7	m, alive 65	AP6, N5, N5, *b*			CTR 47	neg T		
II mit⁻⁻	HCM P-5	m, died 53	AP6, N3, AP6	CO_1		CTR 53	EF 41	AF,CRBBB	
	DCM P-1	m, died 75	N6, Ib	CO_1, *b*		c m	EF 17	AF	
	DCM P-2	m, died 45	AP6, N3	CO_1		CTR 67	EF 12	AF	
III syn⁻	HCM P-8	f, alive 18	N1, N4, N5		$tRNA^{Leu}$		hypok		
	MCM P-1	m, died 19	AP6, N3, N2		$tRNA^{Asp}$		hypok		seiz
IV mit⁻⁻	HCM P-9	m, alive 21	N1	N2	$tRNA^{Thr}$	c m	hypok	**CLBBB**, LVFS red	ment
+syn⁻	HCM P-4	m, died 20		*b*	rRNA	c m	neg T	WPW seiz, ment	deaf, NP,
	DCM P-3	f, transpl 7		CO_1, *b*	rRNA	CTR 68	EF 10	s t	mitr reg, transpl
	HCM P-6	f, died 54	AP6, N3, N2, N4	N1	$tRNA^{Leu*}$	c m		**AV bl**	DM, NP
	FICM P-1	m, died 1	AP6, N3, N2, N1, N2, N2, CO_1, *b*	CO_2, *b*	$tRNA^{Ile}$	CTR 71		pvc bradycardia	arrest, seiz

	HCM P-2	m, died 45	AP6, N3, N2, AP8, N6	N5	$tRNA^{Thr}$	CTR 68	hypok, EF 20		NP
	HCM P-1	m, alive 21	AP6, N3, N1	AP6, N4	$tRNA^{Leu*}$	CTR 58	hypok, EF 45	WPW, s t	DM, deaf, ment

Abbreviations: mit⁻ defined as a base-substitution that causes replacement of a nonconserved amino acid; mit⁻⁻ as replacement of an amino acid conserved among 6 known kinds of mammalian species, with block capital as replacement of the amino acid associated with change of its polarity; syn⁻ as base-substitution in the genes coding for components of the mt protein synthesis system that substitutes the conserved base and/or base-pair among mammals; CO, cytochrome oxidase; *b*, cytochrome *b*; N, NADH dehydrogenase; AP, ATPase; tRNALeu*, transfer $RNA^{Leu(UUR)}$; rRNA, 12S ribosomal RNA; finds, findings; arrhyths, arrhythmias; complts, complications; c.m., cardiomegaly; CTR, cardiothoracic ratio, %; neg T, negative T wave; EF, ejection fraction, %; hypok, hypokinesis; AF, atrial fibrillation; CRBBB, complete right bundle branch block; CLBBB, complete left bundle branch block; LVFS red, reduction of left ventricular fractional shortening during systole; AV bl, atrio-ventricular block; WPW, the Wolff-Parkinson-White syndrome; s t, sinus tachycardia; p v c, premature ventricular contraction; ret pig, retinitis pigmentosa; ment, mental retardation; deaf, deafness; NP, nephropathy; seiz, convulsive seizure; mitr reg, mitral regurgitation; DM, diabetes mellitus; transpl, heart transplantation.

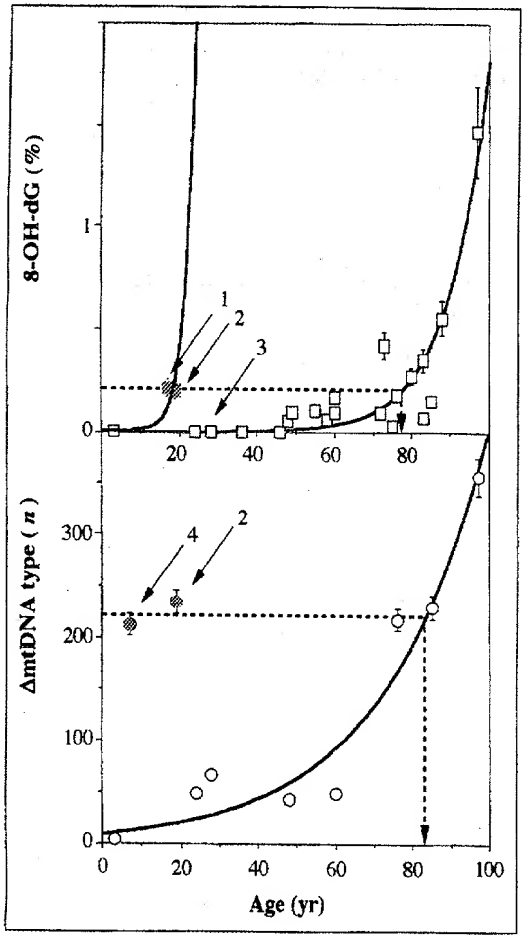

Fig. 12.2. Age-associated correlative increase in the total number of ΔmtDNA type and oxidative damage.

Upper panel: mtDNAs were extracted from autopsied cardiac muscles of 21 human subjects (8 males and 13 females aged 3 to 97 years) without cardiological symptoms obtained at random. Samples of mtDNA were enzymatically hydrolyzed into nucleosides, subjected to precolumn concentration, and analyzed by the microHPLC/MS system.[122] Both selected ion monitoring and ionization MS spectra of 8-oxoG and dG were recorded. The % of 8-oxoG to the total guanine increases exponentially with the ages of subjects [$r = 0.84$]. Overlaid plots are the 8-oxoG % of mtCM patients who died of heart failure at age 17 (female, a closed square indicated by arrow 1) and at age 19 (male, MCM P-1, a closed square indicated by arrow 2)[30] respectively. The 8-oxoG % of a negative control (female, ID119, an open square indicated by arrow 3) harboring nonsevere base-substitutions at a marginal level. An arrow with dashed line indicates that the 8-oxoG % in mtCM patients is equivalent to that of normal subjects of 78 years.

Lower panel: The n of ΔmtDNA types was determined by the total-detection system.[29] 8 out of 21 mtDNA specimens (3 males and 5 females) after the 8-oxoG analyses, shown in the upper panel, are plotted to age. ΔmtDNA type n increases exponentially with the ages of subjects, resulting in a decrease of the wild-type mtDNAs down to 11% with a strong negative correlation with age ($r = 0.89$). Overlaid plots are ΔmtDNA type n of a female DCM patient[31] who received heart transplantation at the age of 7 (DCM P-3, a closed circle pointed by arrow 4), and that of a male mtCM patient who died at age 19 (MCM P-1, a closed circle pointed by arrow 2). Mean ΔmtDNA type n of the positive controls is equivalent to the normal subject of 82 years. Modified from ref. 32.

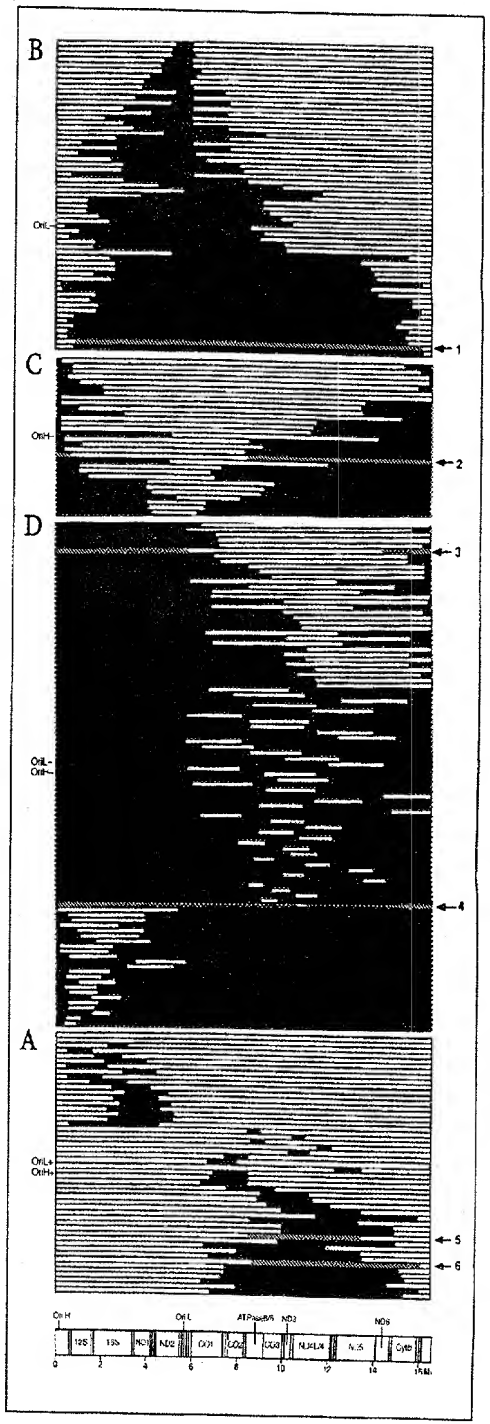

Fig. 12.3. Extensive deletions leading to generation of mtDNA minicircles. The total detection system for the deletions in mtDNA was designed with 180 different primer pairs.[29] In the heart mtDNA of MCM, P-1 having a syn$^-$ mutation in the $tRNA^{Asp}$ gene, 235 types of deletions leading to generation of mtDNA minicircles are detected. Deletions could be classified into four groups: A group of deleted mtDNA (total 48 types) preserving the replication origins (Or) for the H-strand (OrH) and for the L-strand (OrL); B group having OrH but no OrL (total 59); C group having OrL but no OrH (total 31); and D group having neither OrH nor OrL (total 97). Group B, C, and D, either incomplete or complete losses of Or, are regarded as Or$^-$ mtDNA minicircle. Numbered mtDNAs with various sizes of deletion are sequenced including cross over region of the deletion breakpoints. The deleted mtDNA accounted for 84% of the patient's total mtDNA. In contrast to the patient, 67 types of deletions accounting for 23% of the total mtDNA were detected in mtDNA of the syn$^-$ negative control, ID119. Modified from ref. 30.

Table 12.2. Types of mtDNA among subjects

Subject	Sex	Age	Disease	ΔmtDNA Type (n)	Subtype of ΔmtDNA OriL$^+$/H$^+$	OriL$^-$	OriH$^-$	OriL$^-$/H$^-$	ωmtDNA (%)	8-OH-dG per 10^4 dG
A.K.	F	3	VSD	5	4	1	0	0	>99	<1
S.T.	M	24	Accident	49	16	15	8	10	85	<1
N.N.	F	28	Pul. Emb.	67	23	14	8	22	71	<1
Y.I.	F	48	Thymoma	43	13	8	4	18	73	5.9
Y.T.	M	60	Gastric ca.	49	13	23	1	12	71	17.5
Y.Y.	F	76	SAH	218	66	68	37	47	47	18.6
K.A.	M	85	Colon ca.	230	61	64	33	72	58	15.3
H.M.	F	97	Gastric ca.	358	78	88	63	129	11	148
M.K.	F	7	DCM	212	37	58	38	79	48*	38.8*
T.K.	M	19	mtCM	235	48	59	31	97	16	20.1
rho$^+$			normoxia	49	14	15	5	15	80*	<1*
rho$^+$			95% O2	187	35	55	28	69	53*	29.9*

All samples of mtDNA were extracted from autopsied heart muscle, except cultured cell lines.
Abbreviations: ΔmtDNA, mtDNA with deletions; ωmtDNA, wild type mtDNA; Ori, replication origin; F, female; M, male; VSD, ventricular septal defect; Pul. Emb., pulmonary embolism; ca., cancer; SAH, subarachnoidal hemorrhage; DCM, dilated cardiomyopathy;[1] mtCM, mitochondrial cardiomyopathy;[30] rho$^+$, a cultured human cell line, 701.2.8c;[33] *, calculated from the regression formula.[32]

tissues, enhanced tissue oxygenation,[36] and the collapse of the electrochemical proton gradient ($\Delta\mu H^+$) in the form of mitochondrial transmembrane potential ($\Delta\Psi_m$) created by the active ETC.[37]

In mtCM patients, mtDNA mutations, especially those in the genes conferring protein synthesis, are associated with severe heart failure and arrhythmia, implicating apoptosis in cardiomyocytes and in cardiac conduction systems. Recently, the DNA nick-end labeling (TUNEL) on microscopic specimens has been developed to document endonuclease-digested nDNA as the sign of apoptosis. Using this technique, apoptosis was implicated as a possible pathophysiological cause of mtCM/mtDM, as it is prominent in: cardiac myocytes of the failing heart;[38] chronic heart failure;[39] DCM;[40] mtCM harboring serious point mutations;[41] pressure overload-induced cardiac hypertrophy and remodeling in the rat;[42] sinus node cells of patients with complete heart block and fatal arrhythmias;[43] β-cells of insulin-dependent DM model;[44-45] and islets of Langerhans of rats treated with interleukin-1.[46] The molecular genetics and bioenergetics that link the mtDNA mutations and apoptotic cell death, which lead to tissue degeneration and atrophy, have not been fully clarified, however. Evidence suggesting that nDNA decomposition into oligonucleosomes[47] plays a primary role in apoptosis was rejected by the observation of apoptosis by anucleate cytoplasts.[48-50] The active role of reactive oxygen species (ROS) in apoptosis[51-52] suggested by studies on anti-apoptotic protein Bcl-2[53] contrasts with cellular apoptosis under anoxic conditions.[54-55] A survey of recent reports on apoptosis[56-58] enables us to outline the cascade of apoptosis (Fig. 12.4): mtDNA fragmentation, and hence the bioenergetic deficit, results in the collapse of $\Delta\mu H^+$, leading to the opening of $\Delta\Psi_m$-dependent permeability transition (PT)

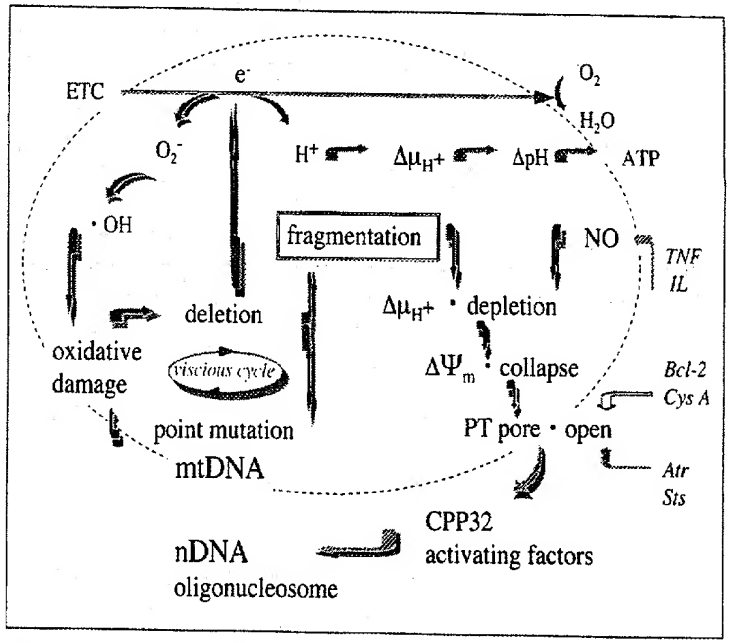

Fig. 12.4. Cascade from mtDNA oxidative damage and fragmentation to nuclear apoptosis. A cascade of cellular apoptosis is schematically illustrated based on the following reports: Occurrence of a particular base substitution in mtDNA of tissues of aging humans.[82] Accumulation of somatic nucleotide substitutions in mtDNA.[27] Extensive tissue oxygenation, focal hyperoxia, associated with mtDNA mutations and with age.[36] Age-associated oxygen damage and deletions of mtDNA in human hearts[20] Accumulation of oxidative damage and mtDNA fragmentation associated with age[32] and with point mutations;[30-31] Age-associated accumulation of oxidative damage and deletion leading to the loss of mt respiratory chain activities.[35-125] Hyperoxia induces an apoptotic cell death associated with fragmentation of mtDNA;[33] Anoxia or inhibition of respiratory chain causes an acute apoptosis.[4,126] NO inhibit CO activity[127] and its synthase (NOS) was extensively induced by cytolytic factors such as TNF[113 114] and IL-1[46,115] leading the target cell to apoptosis; A drop in $\Delta\Psi_m$ is one of the first events in apoptosis.[128] The uncouplers of oxidative phosphorylation or divalent cations cause the collapse of $\Delta\Psi_m$ leading to apoptosis.[57] Oxidants promote PT pore opening[57,129] and reductants prevent;[130] Increase of the gating potential by oxidants and its reversal by reducing agents;[131] Apoptosis is prevented by cyclosporin A, a specific ligand of ANT.[132] The PT induction in response to the ANT ligand atractyloside is inhibited by a specific ANT ligand bonkretic acid or by hyper expression of the anti-apoptotic protein Bcl-2.[57] The overexpression of Bcl-2 retards the necrotic cell death by the respiratory inhibitors such as cyanide.[133] An apoptosis-inducing reagent, staurosporin, results in the PT pore opening and mt swelling eluting mt solutes such as dATP and cyt c that activate an inactive form of an ICE family protease CPP32 to its active form;[58] CPP32 cleaves various substrates including nuclear lamin[134] exposing nuclear DNA to Ca^{2+}-endonuclease digestion[135] and to ladder formation.

The hatched line represents the mt membrane. The single arrows represent the bioenergetic processes. The double arrows represent the cascade of apoptosis. The hatched arrows represent cytosolic processes. Original figure by T.O.

pores, releasing intra-mt apoptotic protease (CPP32, or caspase 3) activating-factors into the cytosol. This is followed by nDNA decomposition and cell death (which could be regarded as bioenergetic cell death). This outline allows the oxidative damage and fragmentation of mtDNA to be linked with the apoptosis cascade.[59]

Comprehensive analyses of mtDNA utilizing the sequence of the entire mtDNA,[26] total detection of ΔmtDNA,[29-32] and oxidative damage of mtDNA[32-34] could reveal a clear correlation between the genotype and the phenotype of mtCM and of senescence.[60]

Point Mutational Genotype and Clinical Phenotype

In 1981 Anderson et al[7] reported the entire nucleotide-sequence of human mtDNA, referred to as the Cambridge sequence (Camb seq).[61-62] In early mutation surveys, comparison of sequenced mtDNA in the patients' somatic cells with the Camb Seq[63] revealed various point mutations that were maternally transmitted through the germ-line cell or acquired during oogenesis. Since then, many point mutations have been identified to be the cause of several degenerative diseases such as Leber's hereditary optic neuropathy (LHON),[61-62] myoclonus epilepsy associated with ragged-red fibers (MERRF),[63,64] mitochondrial encephalomyopathy with lactic acidosis and seizures (MELAS),[65-66] Kearns-Sayre syndrome,[67] Parkinson's disease,[68] some of the primary CMs later named as mtCM,[69-70] Huntington's disease[71] and DM with deafness.[72] However, an early survey of point mutations in mtDNA by restriction analysis or by sequence analysis within limited regions suggested that there is no obvious correlation between point mutation and clinical phenotype. This was based on (i) lack of a real standard sequence from which point mutations diverge; (ii) lack of information to establish point mutational genotypes from entire mtDNA sequences; and (iii) lack of genetic controls to correlate the clinical phenotype. In addition, enthusiastic oversimplification of these relationships lead to inconsistencies; for example, np3243 A→G transition in $tRNA^{Leu(UUR)}$ was first found in MELAS patients[65-66] and named as a MELAS mutation.[25] However, it was also found in mtCM patients without MELAS symptoms[73] and in patients with DM.[72,74]

The following steps were taken to address inconsistencies between point mutational genotype and clinical phenotype:

(1) The Camb Seq was mainly derived from a single placental mtDNA of a normal subject, and also from some regions of mtDNA from HeLa cells. In addition, several ambiguous nucleotides were assumed to be the same as in the bovine mtDNA sequence.[7] Thus, the Camb Seq could not be the real standard sequence for clinical genotype analysis. On the basis of restriction mapping, the Wilson's group[75] concluded that all modern human mtDNA stems from one woman—mtEve, who lived 200,000 years ago. Thus the base-sequence of mtEve should be the standard sequence. However, the base-sequence established by the Wilson group is restricted in only 9% of the entire mtDNA sequence. Hence, much effort in our laboratory has been expended on sequencing the entire mtDNA of modern individuals to infer the standard mtDNA sequence of our common ancestor. We have determined the entire mtDNA sequences of 65 individuals, including 32 patients with mtCM and 10 normal controls among Japanese, American and Australians of European origin by automated sequencing of the entire mtDNA.[26] The accumulated database with one million bp could infer with certainty the unique nucleotide changes of the Camb Seq.[1,28] The total base-sequence of mtEve as the standard

sequence was inferred in this way (Fig. 12.1). Based on the sequence, the phylogenetic tree of the mtCM patients was constructed directly from the entire mtDNA sequence without using incomplete statistical methods to construct phylogenies from a limited number of base substitutions obtained by restriction enzyme assay and/or partial sequencing of the genome.[76] In building the tree, the time-scale was based on the assumption that the rate of nucleotide substitution in human mtDNA is 2×10^{-8} per year per site,[77] i.e., 1.2 nucleotide substitutions per 5×10^3 years per 16.5×10^3 base-pairs in mtDNA. A cascade of sequentially divergent clusters of changes is illustrated in Figure 12.1 (due to space limitations, only the instructive part of the tree is shown). More frequent nucleotide changes are located closer to the standard sequence. The cluster of changes unique to the individual is enclosed with a thick line. The nucleotide-substitutions unique or close to the individual often show heteroplasmic coexistence with the wild type in some tissues.[78] By assuming random segregation of selectively neutral mutants in mammals, it is estimated that it would take at least 20 generations to obtain a pure mtDNA population from a mixed population.[79]

In addition to maternally inherited germ-line mutations, nucleotide substitution has been documented to occur in a single generation of Holstein cows—probably due to a genetic "founder effect" during oogenesis[80]—that is, amplification of one or a few mtDNA molecules as a template will yield one predominant genotype in the mature oocyte that contains 100 to 1,000 times more mtDNA than that found in somatic cells.[81] In addition, a somatically acquired point mutation at np3243 A→G transition has been reported in the cells of an individual.[82] The somatically acquired point mutation was also detected in the cloned skeletal muscle mtDNA from a MELAS patient (10 clones/60 clones). This was significantly higher than in those from normal skeletal muscle (0/60) or in a normal placenta (2/60).[27]

(2) Among the mtCM patients, the major nucleotide-substitutions close to the standard sequence are synonymous. Hence they could be regarded as polymorphism of human mt genome with little pathogenecity (without mark in Fig. 12.1). However, some are nonsynonymous mutations. Mutations especially close to or unique to the modern individuals seem to have significant pathogenicity. With some modifications in the nomenclature from the field of yeast mtDNA,[83] we defined mit^- (marked as *) as a base-substitution causing replacement of an amino acid which is not conserved among six kinds of mammalian species studied (human, bovine, rat, mouse, seal, and whale), mit^{--} (marked as !) as replacement of conserved amino acid, and syn^- (marked as @) as the base-substitution in the genes coding for components of the mt protein synthesis system that substitutes the conserved base and/or base-pair among mammals in tRNA and the conserved base among known biological species in the rRNA gene (rRNA) (the base-substitution of nonconserved base/base pair is marked as #).

(3) Looking at the range of mutational spectra in these patients, we noticed that a classification of patients' point mutations into four kinds of genotype could explain the variety in the seriousness of mtCM, (see Table 12.1). The classification of genotype is based on the nature and combination of these mutations.

(Mit^-) Genotype

This genotype is defined as presence of only mit^- in the entire mtDNA sequence, as listed in Table 12.1. HCM P-3 harbors two mit^{--} one at np8993 T→G in the ATPase subunit 6 gene (ATP6) replacing Leu (conserved in mammals except in the whale)

by Arg, thus changing amino acid polarity, and the other at np9270 C→T in the cytochrome oxidase (CO) subunit 3 gene (CO_3). His symptoms were complicated by neurological findings. HCM P-10, who harbors seven mit^- (Fig. 12.1), has been stable during the past 20 years. HCM P-7 harbors four mit^-, np8584 G→A in the ATP6, np12361 A→G in the NADH dehydrogenase subunit 5 gene (ND_5), np13477 G→A in ND_5, and np15851 A→G in the cytochrome *b* gene (*b*). It is interesting to note that mit^- also exists in mtDNA of the normal controls; e.g., ID 119, who died in an accident at age 28, harbored five mit^- (Fig. 12.1). Thus the possible pathogenecity of each detected mit^- could be regarded as slight. In fact, somatic oxidative damage in this individual's mtDNA is below the detection limit (Fig. 12.2). This is in contrast to MCM P-1, who harbored one additional syn^- at np7579 T→C in $tRNA^{Asp}$ compared to the ID119 genome.[30]

In summary, patients with (mit^-) genotype expressed moderate cardiac hypertrophy and negative T wave in their ECGs. However, their signs and symptoms remained stable.

(Mit^{--}) Genotype

The genotype is defined as mit^{--} with/without mit^- in the entire mtDNA, as listed in Table 12.1. HCM P-5, who harbored one mit^{--} at np6018 G→A in the CO_1 replacing Ala to Thr in addition to three mit^- at np8701, 10398 and 8581 (Fig. 12.1), was clinically diagnosed as DCM associated with complete right bundle branch block (CRBBB), and died of cerebral embolism due to AF. Autopsy showed partial hypertrophy of the left ventricular (LV) wall, leading to progression of HCM to dilatation of LV and dysfunction. DCM P-1, who harbored two mit^{--} at np7041 G→A in the CO_1 replacing Val to Ile and at np15218 A→G replacing Thr to Ala, showed cardiomegaly, diffuse wall thinness, severe hypokinesis of LV, and EF of 17%. DCM P-2, who harbored one mit^{--} at np6402 A→G in the CO_1 replacing Thr to Pro, showed severe hypokinesis of the LV wall and dysfunction with EF of 12%, and he died of heart failure.[73] He had two younger brothers with DCM, one of them died at age 32.

In summary, the patients with (mit^{--}) genotype showed more serious clinical phenotype than those with (mit^-) genotype. All three of the listed patients complained of dyspnea and had atrial fibrillation (AF), atrio-ventricular block (A-V block), or reduced ejection fraction (EF). The mit^{--} in CO seems to cause cardiac hypertrophy and ROS damage of myocardium leading to AF.

(Syn^-) Genotype

The genotype is defined as syn^- with/without the mit^- in the entire mtDNA, as listed in Table 12.1. Patients with this type of mutation showed different signs and symptoms from those with the other genotype. HCM P-8, an Australian of English descent, who had one syn^- at np12308 A→G in $tRNA^{Leu(CUN)}$ (Fig. 12.1), showed little cardiomegaly (heart size within normal limits) and diffuse hypokinesis of the LV wall.[1] Her family history is rather 'malignant', characterized by sudden death. Her twin sister died of heart failure at 5 months after delivery and the autopsy showed CM. Her mother died 6 days after the confinement. One of her uncles died of heart failure at age 16, and the another died at age 43. Her grandmother, who had heart failure, died suddenly in her sleep at age 49. MCM P-1, who harbored one syn^- at np7579 T→C in $tRNA^{Asp}$ with three mit^- (Fig. 12.1) showed short stature, a tremor, and a generalized convulsive seizure at age 10. From the age of 12, he gradually developed heart failure up to grade 4 of the New York Heart Association (NYHA)

standard despite his normal sized heart, and died at his age 19. Diagnosis of mtCM based on postmortem morphological examination of myocardial specimen demonstrated dominant features of apoptotic death of cardiomyocytes; viz., extensive proliferation of abnormally expanded mitochondria, including glycogen granules, with atrophy and breakage in muscle fibers.[30] Genetic analysis demonstrated one additional syn^- compared with his genetic negative control, ID119, that extensively accelerated the oxidative damage and fragmentation of mtDNA equivalent to a normal subject of 80 years (Fig. 12.2). This could explain the development of his heart failure.

In summary, the patients with (syn^-) genotype showed little evidence of cardiac hypertrophy. There were no arrhythmias but diffuse hypokinesis of LV wall was present. In yeast, syn^- results in an impaired system of mitochondrial protein synthesis. Hence, syn^- strains, being pleiotropically deficient in the respiratory and ATPase complexes, are similar to the yeast strains having large deletions.[83] The mtCM patients with this genotype also show pleiotropic symptoms with the defect in protein biosynthesis predisposing cells to pleiotropic dysfunction of electron transfer chain (ETC). Hence the bioenergetic crisis is more severe than mit^{--} (Fig. 12.4). The normal heart size of the patients with this genotype suggests that the hazardous effects of syn^- overcome the hypertrophic response mediated by the growth factors. On the other hand, the pleiotropic defect in the ETC could cause the hypokinesis of the ventricular wall, thus increasing the risk of sudden death, as in the family members of HCM P-8.

($Syn^- + Mit^{--}$) Genotype

The genotype is defined as both syn^- and mit^{--} with/without mit^- coexisting in the entire mtDNA, as listed in Table 12.1. HCM P-9, an Australian of Greek descent, harbors one syn^- at np15928 G→A in $tRNA^{Thr}$ and one mit^{--} at np4917 in ND_2, replacing Asn to Asp changing amino acid polarity (Fig. 12.1). His detailed clinical record is reported elsewhere.[84] He complained of dyspnea and exercise-induced palpitation. A maternal family history of fatal CM is documented. He had severe cardiomegaly and biventricular failure. An echocardiogram revealed dilatation of all cardiac chambers and marked reduction of LV fractional shortening (LVFS). An ECG shows a complete left bundle branch block (CLBBB). HCM P-4, who harbored one syn^- at np827 A→G in $rRNA^{31}$ and one mit^{--} at np15236 A→G in *b* replacing Ile to Val, complained of general fatigue, leg edema, and convulsive seizures. His main complications were short stature, chronic renal failure, mental retardation, ataxia, and sensorineural hearing loss. An ECG showed signs of Wolff-Parkinson-White (WPW) syndrome and giant negative T wave. Short-axis section of the autopsied heart indicated small size of the cavity with extensive hypertrophy of the septum and the LV wall.[31] DCM P-3, a 7 year old female, harbored one mit^{--}, in addition to the HCM P-4 genotype, at np6521 in the CO_1 replacing Ile with Met (Fig. 12.1). Her detailed clinical record is reported elsewhere.[85] Her elder brother with DCM died suddenly at his age 4. She had severely dilated left and right ventricles and regurgitation of both mitral and tricuspid valves. Her cardiothoracic ratio (CTR) increased rapidly from 57% on April 15, 1991, to 63% on June 26, and to 68% on July 22. On July 25, her heart transplantation was performed at the Primary Children's Medical Center in UT, USA. Her excised heart muscle specimen was sent to our laboratory for genetic analysis. HCM P-6, harbored one syn^- at np3243 A→G in $tRNA^{Leu(UUR)}$ and one mit^{--} at np3394 T→C in ND_1 replacing Tyr by His

changing amino acid polarity (Fig. 12.1). She complained of anorexia and general fatigue. Her main complications were second degree A-V block treated with a pacemaker, DM treated with insulin since age 35, and diabetic nephropathy. FICM P-1, who harbored one syn$^-$ at np4317 A→G in tRNA^{Ile} and two mit^{--} at np7673 A→G in the CO2 replacing Ile by Val and at np14927 A→G in *b* replacing Thr by Ala, died at the age of 12 months.[86] He was admitted to a hospital because of general weakness. A chest roentgenogram showed severe cardiomegaly with CTR 71%. He had anemia, metabolic acidosis, and elevated levels of transaminase, lactic dehydrogenase, and creatinine phosphokinase. He suffered from sudden cardiac arrest, then severe arrhythmias, and convulsions and died of heart failure 7 days after admission. HCM P-2, who harbored one syn$^-$ at np15951 A→G in tRNA^{Thr} and one mit^{--} at np13258 A→T in ND5 replacing Ser by Cys (Fig. 12.1), died of heart failure. He had complained of general fatigue and dyspnea on exertion. He had a younger sister with HCM. A chest roentgenogram showed severe cardiomegaly with CRT 68%. Although the endomyocardial biopsy samples showed myofiber hypertrophy and marked disarray, he was diagnosed clinically as DCM from the echocardiogram and cardiac catheterization showing LV dilation, diffuse hypokinesis in LV wall motion, and EF of 20%. HCM P-1, who harbors one syn$^-$ at np3243 A→G in the tRNA$^{Leu(UUR)}$ and two mit^{--} at np8906 A→G in ATP6 replacing His by Arg and at np11084 A→G in the ND4 replacing Thr by Ala (Fig. 12.1), was admitted to a hospital in 1989 because of palpitations and dyspnea. He showed short stature, slight mental retardation, and perceptive deafness. An ECG showed signs of WPW syndrome. An echocardiogram showed marked LV wall thickness and mild diffuse hypokinesis in wall motion. Endomyocardial biopsy samples showed slight hypertrophy and morphological signs of apoptosis; viz., vacuolation of cardiomyocytes, mild fibrosis, abnormal shape of mitochondria, and accumulation of glycogen granules. Laboratory data showed an increase in serum creatinine phosphokinase, lactate and pyruvate at rest. Recently, in 1994, at the age 25, he developed insulin-deficient type of DM. He has been on insulin treatment since then.

In summary, patients with (syn$^-$ + mit^{--}) genotype showed severe and variegated signs and symptoms, and a short life span (Table 12.1). Cardiomegaly with CTR over 60% and arrhythmias are common signs among these patients. Two patients, HCM P-9 and P-6, showed conduction block, and two others, HCM P-4 and P-1, showed WPW syndrome. It will be observed that the mit^{--} in a given patient are synergistic and an additive expression of the patient's clinical symptoms. The excised heart of DCM P-3 retained one additional mit^{--} compared with her syn$^-$ positive control, HCM P-4.[28] FICM P-1 harboring one syn$^-$ and two mit^{--} died at 12 months after the delivery. She had shown variegated signs and symptoms (Table 12.1). The median survival time of six deceased patients with this genotype (21 years—calculated by the method of Kaplan and Meier[87]) is more than 50 years shorter than the average life expectancy of the normal subjects[88] (the life expectancy among Japanese males was 76.25 years and that among females was 82.51 in 1993).

Point Mutational Genotype Relevant to Apoptosis

From the accumulated data, it seems possible that myocardial hypertrophy is triggered by the mit$^-$ and more potently by the mit^{--} leading cardiomyocytes to bioenergetic crisis and to apoptosis. This will be discussed in the following section. Katz[89] suggested that myocardial overload initiates an unnatural growth response that appears to shorten cardiac myocyte survival, possibly because the same growth factors that mediate the hypertrophic response of the adult heart can also induce apoptosis. Experimentally, cardiac hypertrophy and remodeling is initiated by a wave of apoptosis of rat cardiomyocytes.[42] The occurrence of severe arrhythmias, prominent among mtCM patients with mit^{--}, is consistent with the finding[43] that apoptosis is a possible cause of gradual development of complete heart block and fatal arrhythmias associated with absence of the AV node, sinus node, and internodal pathways that are destroyed by a non inflammatory degeneration with no abnormal fibrosis or infiltrate. In this respect, it is noteworthy that two (mit$^-$) genotype patients with the early onset of HCM at younger age had harbored the mit$^-$ in CO (Table 12.1). Mit^{--} in CO was associated with severe cardiomegaly, heart failure, and arrhythmias. The mit^{--} in CO with the additional syn$^-$ was associated with early onset of severe cardiomegaly in pediatric patients, DCM P-3 and FICM P-1. These data suggest that an increase in the release of ROS from defective CO may accelerate oxidative damage and apoptosis.

Progression of HCM to DCM during the course was observed in the cases of HCM P-2 and P-5. The syn$^-$ with mit^{--} triggers degenerative changes in other organs, especially those with postmitotic cells; viz., convulsive seizure, mental retardation, deafness, nephropathy, and DM, as listed in Table 12.1. HCM P-6 and P-1, who harbor the syn$^-$ at np3243 A→G, could be included in diabetic CM.[6] The np3243 syn$^-$ has been reported to be common among the patients with DM with deafness,[72] and patients with insulin-deficient type of DM.[74] The np3243 syn$^-$ seems to have a more moderate pathogenecity than other syn$^-$ because it shows relatively frequent occurrence and relatively late onset of DM in mtCM patients—HCM P-6 at age 35 and HCM P-1 at age 25. The onset of their DM is consistent with the clinical observation that the onset of DM with deafness associated with this syn$^-$ ranges from age 20 to 40, which is in-between those of type I and II DM.[5] These facts are in agreement with the finding[90] that cytokine induces apoptotic cell death in a mouse pancreatic beta-cell line, and the death is prevented by Bcl-2.

Somatic Mutations

Concerning deletion, Grivell in 1989[91] stated that, curiously, there is no obvious correlation between the severity of the clinical symptoms or biochemical abnormality and either the location of the reported deletion or the number of deleted genes. In retrospect, the anomaly seems to be due to limited survey of deletions using a particular primer-pair. This problem was solved by the total detection of mtDNA deletions.[29-32]

With respect to somatic mutations, the age-related cumulative increase in oxidative damage in heart mtDNA correlates closely with the increase in the number of deletions.[1,20] Accumulated somatic mutations in mitochondrial genes lead cells to: bioenergetic crisis, death under physiological conditions, and tissue degeneration and atrophy. The mechanism predicts that somatic mutations would satisfy the following: (i) The mutations arise afresh with each generation and accumulate in an age-dependent manner; (ii) The absolute level of accumulated mutations is accountable for age-related decline of mitochondrial function and bioenergetic deficit; and (iii) The mutations correlate closely with oxidative damage and cell death.

Deletions of mtDNA

Soon after the use of PCR became common, multiple populations of ΔmtDNA were detected in the myocardium.[92] Using PCR, we detected[93] that multiple forms of ΔmtDNA pleioplasmically coexist with wild type (ω) mtDNAs in a tissue. Quantitative data on a PCR-detectable ΔmtDNA in individuals of various ages indicate that there are four orders of magnitude fewer ΔmtDNAs in infants compared to old individuals.[94-95] A newborn harbors extremely low amount of commonly observed deletion (5 kbp) in adults.[16] It is not detected in corresponding fetal tissues.[17] Hence, PCR-detectable multiple forms of ΔmtDNA seem to arise afresh with each generation, satisfying qualification i).

On the other hand, a cell harbors hundreds of mitochondria and mtDNA copies, and the fractional concentration of each ΔmtDNA detected by the conventional PCR using a single primer-pair is usually 0.01-0.3% of the total mtDNA.[96-100] The TD system[29] that enables us to detect all possible ΔmtDNA over 0.5 kilo-bp was applied to mtDNA specimens from normal hearts of various ages.[32] We detected as many as 358 types of ΔmtDNAs, including 280 types of "minicircles" that lack either one replication origin (Ori) or both as shown in Fig. 12.3. Among normal subjects, the ωmtDNA fragmentation into ΔmtDNAs is demonstrated to increase progressively with age and is correlated with the oxidative damage (see Fig. 12.2). Similar fragmentation, rearrangement, and depletion of ωmtDNA was detected by using a long PCR in skeletal muscle of older subjects.[101] Thus, PCR-detectable forms of ΔmtDNA satisfy the qualification ii). In the mtCM patients harboring severe point mutations in mtDNA[30-31] similar oxidative damage and mtDNA fragmentation at age of 7 to 19 is documented to be premature aging equivalent to normal subjects of age over 80 (Fig. 12.2), satisfying qualification iii).

Oxidative Damage

As early as the time of discovery of oxygen, Priestley[102] commented that 'oxygen might burn the candle of life too quickly.' However, it took a long time to elucidate the mechanism of this toxicity leading to cell death (Fig. 12.4).

In ETC, the majority of electrons reduce molecular oxygen in the terminal oxidase, CO, forming H_2O. However, a small number of electrons generated from ETC performs one-electron or two-electron reduction of oxygen forming ROS. In 1924, Warburg[103] suggested that oxygen when activated by CO, undergoes four-electron reduction forming water without H_2O_2 formation. However, Michaelis[104] proposed that the oxidation of bivalent organic molecules proceeds in two obligatory univalent steps, the intermediate being a free radical; viz., one-electron reductant, O_2^-, and two-electron reductant, H_2O_2. It was, therefore, a matter of great interest to discover the ETC as the production site. In 1975, on the basis of optical studies on oxy- and peroxy-CO by Chance et al,[105] it became clear that the intermediates remain within the active site of CO, until the final four-electron reduction of oxygen to form water is achieved, probably for protection against cellular intoxication. From general properties of the mitochondrial generation of H_2O_2, and the effect of hyperbaric oxygen, it was postulated[106] that besides the well-known flavin reaction, formation of H_2O_2 may be due to interaction with an energy-dependent component of ETC at the cytochrome (cyt) *b* level. These findings indicated that the active sites of the complex IV and III, cyt *a* and *b*, play a crucial role not only in the cellular energy production, but also in protection against cellular oxidative damage. Hence, the genetically defective cyt *a* and *b* could may affect cellular viability. Accordingly, severe DCM or HCM is observed in mtCM patients, who harbor mit^{--} in CO and *b* and/or syn^- in tRNA that affect the translation of the cyt genes, as listed in Table 12.1.

From the above mechanism of oxygen reduction, the elution of electrons from ETC and the generation of ROS can be deduced not only from the genetic defect but also from physiological attenuation of the cellular redox state. Hyperoxia[107] increases H_2O_2 release by lung mitochondria, because of too much oxygen supply over and above enzymatic capability to dispose off ROS. Skulachev[37] pointed out that mammalian uncoupled respiration or noncoupled respiration in plants is an effective way to prevent oxidative damage and cell death. Boveris and Chance[106] demonstrated that H_2O_2 production by animal mitochondria, negligible in active respiration (State 3) or in the presence of uncouplers, becomes quite measurable in resting respiration (State 4). State 4 increases reduced electron carriers such as flavins, NADH oxidoreductase, CoQ, cyt *b*, and nonheme iron proteins[108-110] which are the main targets for one-electron oxidation by oxygen. State 4 mitochondria from rat liver or from pigeon heart generate about 0.3-0.6 nmol H_2O_2/min per mg of protein.[106] This H_2O_2 generation represents approximately 2% of the total oxygen utilization under these conditions. Thus, during the total life of an individual, a large amount of the redox energy is consumed for the generation of ROS. In addition, we found[111] empirically that cyt c, an essential component of ETC, is 20 times more efficient as a catalyst than ferrous ions in promoting the formation of the most reactive oxygen radical—hydroxyl radical (•OH) by the Fenton reaction. Thus ETC is the major source of •OH production (Fig. 12.4). The •OH production was demonstrated by interaction of O_2^- with nitric oxide (NO)[112] of which synthase (NOS) was extensively induced by cytolytic factors such as tumor necrosis factor (TNF)[113-114] and interleukin-1β (IL-1)[46,115] targeting cells to apoptosis.

In this respect, •OH is not merely a byproduct of the mitochondrial respiration, but plays an important bioenergetic role in causing physiological cell death and in eliminating unwanted/transformed cells. The mtDNA is located inside the mitochondrial inner-membrane where ROS is continuously leaked out from the respiratory chain.[116] Hence it is susceptible to attack by ROS,[117] despite cellular defenses against. During evolution, human mtDNA is downsized (to 1/5 when compared from yeast), and lost its intron. Perhaps due to these reasons, human mtDNA is extremely fragile and susceptible to oxidative damage when compared to unicellular organisms.

The underlying mechanism for the deletions may be the double-strand breaks.[118] The 8-hydroxy-deoxyguanosine (8-oxoG),[119] a hallmark of oxidative damage to DNA, is rapidly excised from nDNA and excreted.[120] However, 8-oxoG accumulate in mtDNA with age specially in the postmitotic tissue leading to random point mutations, double-strand and single-strand breaks and large deletions. A study[121] of mutagenesis of 8-oxoG demonstrates that a synthetic proto-oncogene containing 8-oxoG induces point mutations during replication (see chapters 1 and 6 in this volume for more details on the mechanisms of mutation). A defective electron-transport chain encoded by the mutated mtDNA may enhance ROS production, resulting in increased accumulation of 8-oxoG. Such a vicious cycle of oxidative damage and mutations in mtDNA seems to result in those changes becoming synergistic and exponential, as illustrated in Figure 12.4.

In human heart mtDNA specimens, quantitative determination of a single ΔmtDNA with 7.4 kbp deletion demonstrated an age-related progressive increase.[20] This correlates closely ($r = 0.93$) with the accumulation of 8-oxoG[122] up to 1.5% at age 97. A similar accumulation in 8-oxoG was reported in human brain mtDNA (up to 0.87%) at age 90.[123] The TD system revealed that a progressive age-related increase in the total number (n) of ΔmtDNA correlates with accumulation of 8-oxoG in the heart mtDNA (Table 12.2, Fig. 12.2) reflecting a long-term accumulation of oxidative damage during human life. A remarkable mirror image of the size distribution of ΔmtDNA[32] (Fig. 12.3) and a strong linear correlation ($r = 0.97$) between minicircles and ΔmtDNA preserving both replication origins (Table 12.2) suggests random occurrence of ΔmtDNA without any preferential site. Thus, it seems reasonable to presume that random double-strand separation by accumulated 8-oxoG,[20] single-strand breaks by •OH attacks[118] and rejoining of mtDNA may be the mechanism for generation of hundreds of ΔmtDNAs. Experimentally, these changes in mtDNA correlate with the decline in the ETC activity in the laboratory animals.[34-35] An extensive oxygenation of skeletal muscle indicating mitochondrial dysfunction is demonstrated noninvasively in senescent individuals and patients with mtCM and/or myopathy harboring point mutations.[36] Similarly, reduced oxidative metabolism is reported in the cortex of Alzheimer type dementia.[124] Therefore, the vicious cycle of progressive oxidative damage, fragmentation of ωmtDNA, defective ETC, and relative tissue hyperoxia seems to result in those changes to be synergistic and exponentially associated with age (Fig. 12.2). The mtDNA diseases may, perhaps, be regarded as premature aging of tissues where oxygenation and the mtDNA fragmentation are abnormally accelerated by synergistic factors. Interestingly, premature aging with the bioenergetic cell death can be mimicked by cultured cell lines under hyperoxia conditions.[33] The exposure of a cultured fibroblast cell line (rho^+) under oxygen stress leads to apoptotic cell death with mtDNA fragmentation whereas the derivative cells (rho^0) lacking mtDNA were relatively unaffected. These results indicate that human mtDNA is extremely susceptible to oxidative stress leading to mutations. In the context of mtCM, inherited/acquired mutations in mtDNA may result in the biosynthesis of abnormal subunits of energy producing system and in accelerated /uncontrolled production of ROS leading to cell death. The genetic analyses on autopsied myocardia of pediatric patients have documented the increased oxidative damage and ΔmtDNA leading to fragmentation of mtDNA into hundreds of kinds of minicircles[28,30] (Table 12.2, Fig. 12.2). These deleterious mutations could cause apoptotic cell death (Fig. 12.4), abnormal growth of cardiac myocytes, and heart failure.

Conclusion and Perspective

Comprehensive analyses of oxidative damage, inherited point mutations, and total deletions reveal the mutational genotype unique to individuals with mtCM. The analyses indicate that the types and combinations of point mutations decide the severity of oxidative damage. There is a definite correlation between the point mutational genotype and the phenotype of a patient. Survey of point mutations will be useful for genetic diagnosis predicting the patients' life span and for the management of patients using cardiac transplantation and/or gene therapy.

This review of total mtDNA mutations reveals that mtDNA is fragile to oxidative stress resulting in an inactive mitochondrial energy producing system and cellular bioenergetic crisis and cell death. MtDNA may provide the endogenous

link in the cascade of cell death under physiological conditions without vascular involvement. Fragmentation of mtDNA leads to cellular bioenergetic crisis due to defective respiratory chain,[36] to the collapse of $\Delta\psi_m$,[57] to the release of the apoptotic protease activating factors into cytosol,[58] to cell death, to tissue degeneration and atrophy, and to heart failure. Understanding this apoptotic cascade will enable us to prevent mitochondrial diseases.

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